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14. ABSTRACT
The long-term objective of this effort is to develop a generic gene shuffling-based technology to rapidly screen libraries of 1010 proteins/peptides encoded by DNA libraries, for identifying biomolecules that can intercept both existing and emerging organophosphate-based chemical warfare nerve agents (CWNA). The specific milestones for year 1 were (a) Proof of concept for the proposed technology; (b) Generation of 4-6 libraries based on recombinant PON1 and PON3 based on gene shuffling and random mutations (c) synthesis of OP model compounds. In general, all 1st year milestones were met and well beyond, specifically (i) the generation of recombinant PON3 variants and (ii) generation of recombinant PON1 variants, two of them (3B3 and 2B4) were overexpressed, purified and found to have a 72- and 13-fold increased kcat/Km relative to the wild-type like enzyme toward a cyclosarin model compound. Notably the increased activity of these variants seems to be highly selective, e.g. the activity of the two variants with the diethyl phosphoryl analog DEPCyC shows one to be lower and the other to be only 3-fold higher than the wild-type PON1. Relevance: this technology is envisaged to provide rapid discovery of pretreatment and post challenge therapeutic drugs against existing and emerging CWNA threats and will shorten the time from emergence of a threat to identification of potential counter-measures to a few days or weeks.

15. SUBJECT TERMS
Directed evolution, X-ray crystallography, organophosphates & bioscavangers

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Introduction

The long-term objective of this effort is to develop a generic gene shuffling-based technology to rapidly screen libraries of 10^{10} proteins/peptides encoded by DNA libraries, for identifying biomolecules that can intercept both existing and emerging organophosphate-based chemical warfare nerve agents (CWNA). Enzymes identified in these screens should be capable of catalytically neutralizing the target agent under physiological conditions, thereby providing a basis for development of a new generation of therapeutic agents against CWNA. The major milestone is to integrate established components of enhanced molecular evolution techniques so as to provide a means of miniaturizing existing low-throughput assays, thereby dramatically increasing both sensitivity and throughput. Micro beads will be coated with multiple copies of recombinant human AChE (the CWNA physiological target). Genes of interest will be attached to the same beads. Gene libraries will be obtained by random mutagenesis of several genes that encode enzymes capable of hydrolyzing OPs (e.g., organophosphate hydrolases of both bacterial and mammalian origin, and a repertoire of AChEs, *e.g.* other vertebrate AChEs, insect AChEs). Beads coated with AChE and the corresponding genes will be compartmentalized in ~5fL emulsion droplets, and single genes transcribed and translated in individual droplets. The expressed biomolecules will be allowed to intercept the CWNA, preventing interaction with its target (*viz.* AChE), and genes that code for an effective interceptor will be isolated. Uniquely, this screen is for directly detoxifying the CWNA, not simply for binding it, thus allowing identification of biomolecules that prevent its action by degradation at the desired rate. This technology is envisaged to provide rapid discovery of pretreatment and post challenge therapeutic drugs against existing and emerging CWNA threats and will shorten the time from emergence of a threat to identification of potential counter-measures to a few days or weeks. Once developed this technology can be extended to identification of interceptors for vesicants, pulmonary agents, metabolic/cellular poisons and biological warfare agents

Body

I. Specific Aims

1. The development of high-throughput-assays for OP hydrolase variants exhibiting high specificity factors and turnover.

2. Provision of proof-of-concept for the proposed core technology employing directed evolution of new recombinant PON and AChE variants.

3. Isolation of interceptors for G- and V-type nerve agents, and expression in soluble form.

4. Design, generation and selection of 2nd generation libraries for V- and G-type agents.

5. Large-scale production of selected enzyme candidates, and their kinetic, structural and pharmacological evaluation

6. Establishment of "off-the-shelf" libraries for rapid identification of antidotes against emerging future threats

II. Significance to the goals of Counter ACT

The proposed approach opens new opportunities for rapid identification, characterization and implementation of novel countermeasures against CW agents. It will significantly decrease the time interval between the appearance of a new threat and discovery of potential antidotes to counteract it. The major benefits will be one or more products capable of efficient catalytic hydrolysis of G- and V-type nerve agents, as well as gene libraries derived from existing enzymes that can be used "off-the-shelf" to isolate new protein variants for almost any nerve agent or toxic industrial chemical serving as a target for the screen.

III. Year 01 Milestones

In general all 1st year milestones were met, and well beyond.

01 Milestone #1: Provision of proof-of-concept for the proposed core technology by establishment of an emulsion-FACS screening system, leading to miniaturization of existing low-throughput assays to increase sensitivity and throughput by over 1000-fold.

a) We have developed a screen for enzyme variants with increased OPH activity, based on sorting emulsion droplets by FACS. We demonstrated this screen by the model selection of

PON1 variants with increased OPH activity. To do so, we used the fluorogenic phosphotriester DEPCyC (Aharoni et al. 2004), and demonstrated the FACS separation of variants with increased activity towards DEPCyC from a large excess of inactive variants. A typical selection (see Appendix Figure 1) involved the sorting of ~107 cells, each carrying a single enzyme encoded plasmid. The enrichments observed were ≥50-fold, with a recovery of ~50%. The sensitivity achieved at this stage is reasonable but not high (separation of enzyme variants differing in their k_{cat}/K_m values by ~100-fold) and we do aim at improving this range. Given that the standard screens using automated liquid handling are generally performed at a scale of 103-104 variants (up to hundred 96-well plates) this development represents an increase of >1000-fold in screening capacity.

b) We have also pursued the selection of PON1 libraries (see below) with the fluorogenic cyclosarin analogue (CMP) (Amitai et al. 2007a), but obtained low enrichments. Further work indicated that this failure is due to the application of the PON1-GFP fusion that blurred the discrimination of single cell events. Our current work is aimed at selecting libraries of PON1 expressed on its own, rather then as GFP fusion. Once this screen is set up, we will select libraries based on the 'neutral drift' repertoires, as well as various 'naive' PON1 libraries (see below).

01 Milestone #2. Generation of 4-6 different gene libraries, based on recombinant PON1 and PON3, utilizing both gene shuffling and random mutations at varying rates.

We generated 4 different libraries, as detailed below. We have also screened few of these libraries with very encouraging preliminary results (see below). The generated libraries include:

a) *Randomized PON1 libraries*. Starting from a recombinant PON1 gene (variant G3C9; (Aharoni et al. 2004), errorprone PCR was applied at a set of different conditions, and four libraries were generated. The mutated genes were recloned, and the libraries electropoarted at high efficiency (≥105 individual transformants). Characterization of the resulting libraries indicated an average mutation load of ranging from 1.88 up to 6 mutations per gene in the library with highest mutation rate.

b) *A 'neutral drift' PON1 library*. Our lab is developing a novel, and far more effective protocol for directed evolution, by which the target gene is taken through several rounds of intensive mutagenesis and selection for its original function. The gene repertoires that result from

this neutral drift are then screened for the new function. Previous work in the lab has generated a 'neutral drift' repertoire of ~350 PON1 variants fused to GFP (to ensure that expression levels are not reduced), and demonstrated that, despite the miniscule size of this repertoire, it carries many variants with new phenotypes, including variants with increased OPH activity (Amitai et al. 2007b). In conjunction with this project, the neutral drift library was screened using the fluorogenic cyclo-sarin analogue (CMP) (Amitai et al. 2007a) and the pinacolyl analogue PMP (Amitai et al. 2007a) (structures are provided in the Appendix). This screen resulted in the identification of at least two PON1 variants that exhibit marked improvement with these OP substrates (see below).

c) *Expanded 'neutral drift' PON1 libraries*. The 350 neutral PON1 variants described above were recloned while removing the GFP fusion, thus yielding a new library in which the expression level and activity of PON1 was markedly increased. By appending the H115W library on this entire repertoire of neutral variants, a new library was generated for the isolation of variants that can hydrolyze P-S OPs (or V-type OPs) (see below).

d) *PON3 libraries*. A library of PON3 gene variants was generated by family shuffling of four mammalian PON3 genes. The library was characterized and shown to have, on average, ~10 randomly spread crossovers per gene. The library was screened for PON3 primary activity as lactonase, using a chromgenic lactone substrate (TBBL). Variants showing increased expression, and no significant alterations of substrate selectivity with respect to wildtype PON3, were used as the starting point for a 2nd generation library. Screening and shuffling was repeated for another 2 rounds, thus leading to the isolation of novel recombinant PON3 variants described below.

01 Milestone #3. Synthesis of four fluorogenic OP analogs is planned

a) V-type analogs for screening. To enable the evolution of PON1 variants capable of hydrolyzing V-type OP, we have synthesized and characterized Amiton – a P-S analog the hydrolysis of which can be detected with chromogenic and fluorogenic probes (see Appendix, Fig 2.). We have subsequently tested the activity of wild-type PON1, and of all PON1 mutants available in our laboratory, to hydrolyze Amiton, and found that, only the H115W mutant (Yeung et al. 2005) is capable of hydrolyzing Amiton. Although hydrolysis proceeds at low rates (1.7X10-4 units/mg enzymes, relative to 8.26 units/mg for paraoxon), this mutation can serve as

the basis for further improvements. We therefore generated libraries of PON1 based on the H115W mutation, and will screen them for Amiton hydrolysis in the nearest future.

b) Flourogenic G-type analogs. We have explored the feasibility of various fluorophores, and planned the synthesis of several OP analogs that can be applied for the FACS-emulsion high – throughput screen described above. The details are provided in the Appendix (Figs 3&4). However, the main obstacle in pursuing this synthesis has been the availability of starting materials (many of which are unavailable due to security regulations, and the rest cannot be delivered by air). Nevertheless, we anticipate that the essential starting materials will become available in two months, and the synthesis of the planned OP analogues completed within six months. To avoid delays in the progress of the PON1 directed evolution projects, we have been collaborating with Dr. Gabi Amitai (Israel Institute of Biological Research in Ness Ziona) who provided the CMP and PMP analogs on a collaborative basis (Amitai et al. 2007a).

01 Milestone #4. Additional results beyond the committed milestones.

a) Recombinant PON3 variants. Following the screening of the PON3 libraries described above, a series of new PON3 variants has been isolated. In oppose to wild-type PON3s, these express in *E. coli* in a soluble and functional manner, and at relatively high levels (≥10mg per liter culture). The variants were purified and their enzymatic properties studied in detail. These studies indicated that their substrate selectivity largely follows that of wild-type PON3, and thus these variants represent the first bacterially expressed PON3 with wild-type like features.

b) Recombinant PON1 variants. The screen described above led to the isolation of two new PON1 mutants with increased OPH activities. These were over-expressed, purified and characterized in detail. This analysis indicated ~72-fold for 3B3 [N41D, S110P, L240S, H243R, F264L, N324D & T332A] and ~13-fold for 2B4 [D78A, V127A, L240S, H243R, F264L & T332A] increase in k_{cat}/K_m (relative to wild-type PON1) towards the cyclosarin analog CMP. The variants increase seems to be highly selective – *e.g.* their activity with phosphotriesters (DEPCyC) is about 3-fold higher for 3B3 while 3-fold lower for 2B4 than the wild-type PON1.

Key Research Accomplishments

- Development of a screen for PON1 variants with increased OP activity, based on sorting emulsion droplet by FACS.
- Development of a novel neutral drift PON1 library
- Synthesis of amiton, a P-S model compound for VX
- Selection of PON1 libraries with flurogenic cyclosporine analog (CMP)
- Generation of 4 different libraries and screening them with flurogenic OP model compounds
- Isolation, overexpression and purification of two variants with enhanced hydrolysis towards the methylphosphonyl model of the cyclosarin analog CMP

Reportable Outcomes

Manuscripts:

1. Harel, M., Brumshtein, B., Meged, R., Dvir, H., Ravelli, R.B.G., McCarthy, A., Toker, L., Silman, I., and Sussman, J.L. 2007. 3-D Structure of serum paraoxonase 1 sheds light on its activity, stability, solubility and crystallizability. Arch. Ind. Hyg. Toxicol. 58: 347-353.

1st Annual CounterACT Network Research Meeting 2007
Abstracts:

1. J.L. Sussman, Y. Ashani, O. Khersonsky, R. D. Gupta, I. Silman & D. S. Tawfik "Directed Evolution and Structural Analysis of Recombinant PON and AChE variants" - Poster at the 1st Annual CounterACT Network Research

Conclusions

The model OP compounds together with the screening approach that we developed and the libraries that were generated established our methodologies for future screening and identification of promising PON variant candidates. The promising candidates will be tested at ICD with threat agents.

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Aharoni, A., Gaidukov, L., Yagur, S., Toker, L., Silman, I., and Tawfik, D.S. 2004. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc. Natl. Acad. Sci. USA* 101: 482-487.

Amitai, G., Adani, R., Yacov, G., Yishay, S., Teitlboim, S., Tveria, L., Limanovich, O., Kushnir, M., and Meshulam, H. 2007a. Asymmetric fluorogenic organophosphates for the development of active organophosphate hydrolases with reversed stereoselectivity. *Toxicology* 233: 187-198.

Amitai, G., Devi Gupta, R., and Tawfik, D.S. 2007b. Latent evolutionary potentials under the neutral mutational drift of an enzyme. *HFSP Journal* 1: 67-78.

Yeung, D.T., Lenz, D.E., and Cerasoli, D.M. 2005. Analysis of active-site amino-acid residues of human serum paraoxonase using competitive substrates. *FEBS J.* 272: 2225-2230.

Appendices

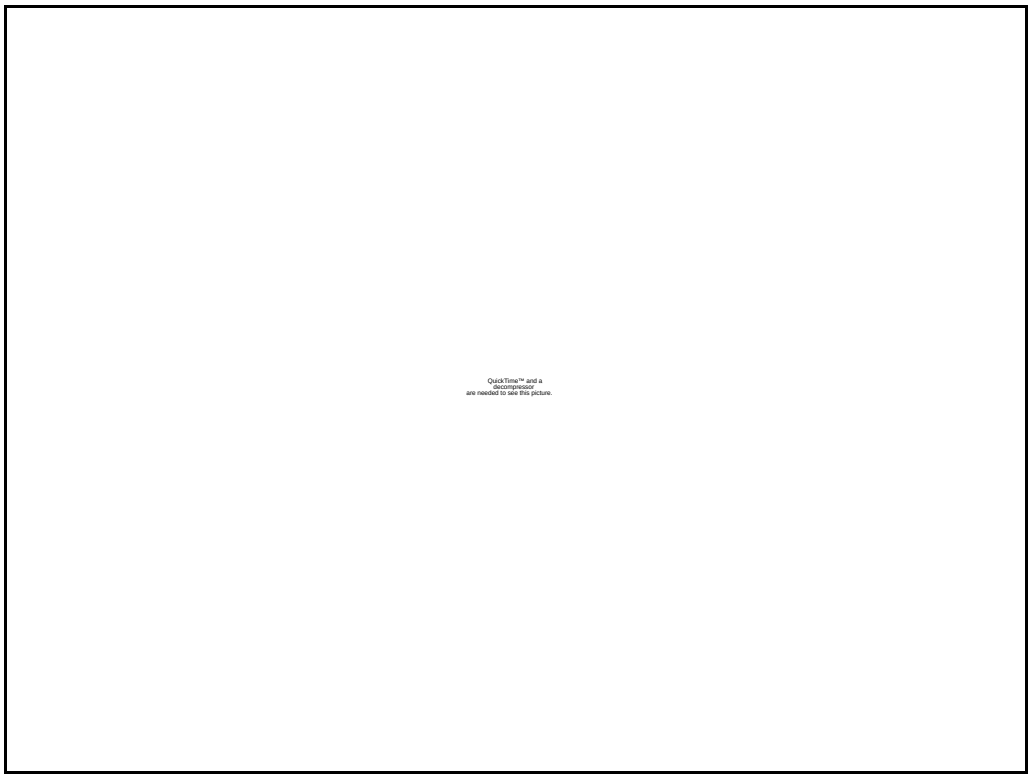

Figure 1. Demonstrating the capabilities of the OPH screen. A mixture of bacteria carrying a plasmid encoding an improved PON1 variant 4.27 (k_{cat}/K_m for DEPCyC = $1.4X10^6$; (Aharoni et al. 2004)), or a plasmid encoding wild-type PON1 (k_{cat}/K_m for DEPCyC = $0.9X10^4$), were mixed at 1:100 ratio. Approximately 10^9 E. coli cells were compartmentalized in emulsion droplets of a w/o emulsion. PON1 was fused with GFP at its C-terminal for increased sorting rate and enrichment. The fluorogenic substrate is added (through the oil phase), and the w/o/w emulsion is formed by emulsification of the primary w/o emulsion, enveloping the aqueous droplets with an intermediate layer of oil and providing an external aqueous phase. Compartments containing the fluorescent product are sorted by FACS, and the cells imbedded in them, together with the gene encoding the enzyme of interest, are isolated. (1) Histograms of the GFP emission (FITC-A) for all the population of droplets. (2) Histograms of the fluorescence at 450nm corresponding to the formation of methyl cyano coumarin (Violet1-A). (3) Events gated in R1 correspond to droplets that contain GFP expressing cells.(4) Dot plot for Violet 1A/FITC-A. R2 gate corresponds to 0.1% of total population, which was sorted and further screened in 96-well plates. Here, we got 54-fold enrichment.

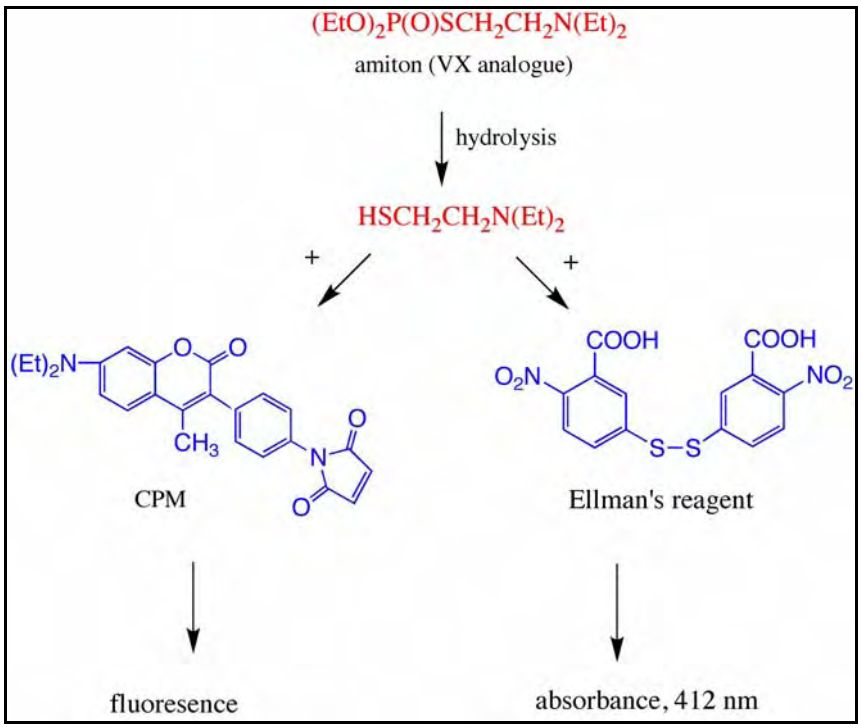

Figure 2. Analytical pathways to monitor enhanced hydrolysis of amiton (VX analog) for screening of PONs capable of hydrolyzing P-S-alkyl bond.

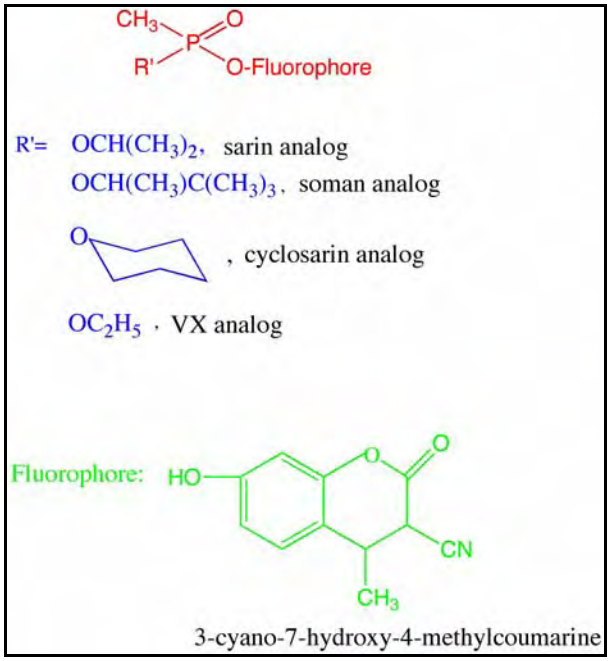

Figure 3. Fluorescent OP analogs of soman and cyclosarin. See figure 4 for planned synthesis pathways

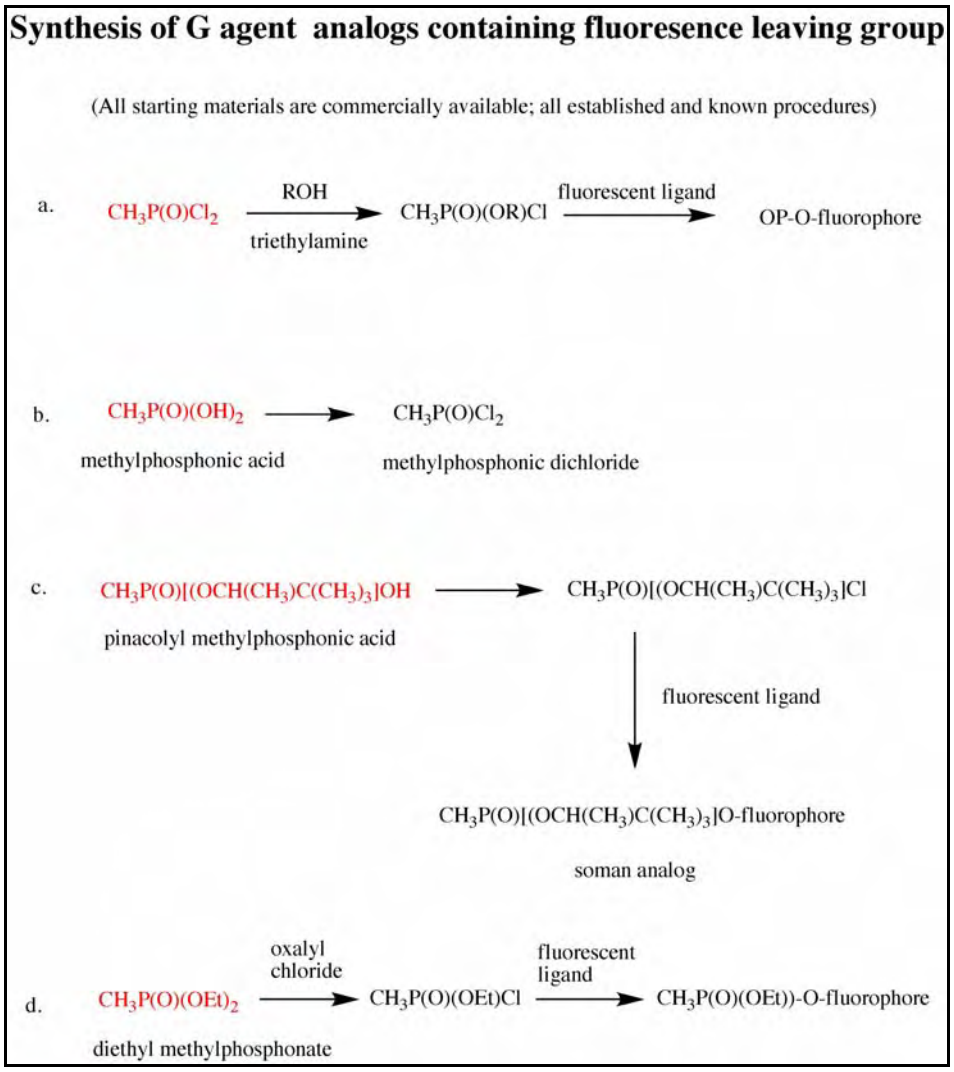

Figure 4. Planned synthesis pathways of fluorophore-containing nerve agent analogs of the G-series.